The effect of dietary addition of nitrate or increase in lipid concentrations, alone or in combination, on performance and methane emissions of beef cattle

Duthie, C-A; Troy, SM; Hyslop, JJ; Ross, DW; Roehe, R; Rooke, JA

Published in:
Animal

DOI:
10.1017/S175173111700146X

First published: 13/07/2017

Document Version
Peer reviewed version

The effect of dietary addition of nitrate or increase in lipid concentrations, alone or in combination, on performance and methane emissions of beef cattle

C-A. Duthie[1], S.M. Troy[1,a], J.J. Hyslop[2], D.W. Ross[1], R. Roehe[1] and J.A. Rooke[1]

[1]Beef and Sheep Research Centre, SRUC, West Mains Road, Edinburgh EH9 3JG, UK

[2]Beef and Sheep Select, SAC Consulting Ltd., SRUC, Kings Buildings, West Mains Road, Edinburgh, EH9 3JG, UK

[a] present address: Blueball, Tullamore, Co. Offaly, Ireland.

Corresponding author: John Rooke. E-mail: john.rooke@sruc.ac.uk

Short title: Methane mitigation strategies for finishing steers

Abstract

Adding nitrate to or increasing the concentration of lipid in the diet are established strategies for reducing enteric methane (CH_4) emissions, but their effectiveness when used in combination has been largely unexplored. This study investigated the effect of dietary nitrate and increased lipid included alone or together on CH_4 emissions and performance traits of finishing beef cattle. The experiment was a 2 × 4 factorial design comprising two breeds (AAx, cross-bred Aberdeen Angus; LIMx, cross-bred Limousin steers) and four dietary treatments (each based on 550 g forage: 450 g concentrate /kg DM). The four dietary treatments were assigned according to a 2 x 2 factorial design where the control treatment contained rapeseed meal as the main protein source which was replaced either with nitrate (21.5 g nitrate/kg DM); maize distillers dark grains (MDDG, which increased diet ether extract from 24 to 37 g/kg DM) or both nitrate and MDDG. Steers (n = 20 /dietary treatment) were allocated to each of the four treatments in equal numbers of each breed with feed offered *ad libitum*. After 28 days adaptation to dietary treatments, individual animal intake, performance and feed efficiency were recorded for 56 days. Thereafter, CH_4 emissions were measured over 13 weeks (six steers / week). Increasing dietary lipid did not adversely affect animal performance and showed no interactions with dietary nitrate. In contrast, addition of nitrate to diets resulted in poorer live-weight gain ($P<0.01$) and increased feed conversion ratio ($P<0.05$) compared with diets not containing nitrate. Daily CH_4 output was lower ($P<0.001$) on nitrate-containing diets but increasing dietary lipid resulted in only a non-significant reduction in CH_4. There were no interactions associated with CH_4 emissions between dietary nitrate and lipid. AAx steers achieved greater live-weight gains ($P<0.01$), but had greater DM intakes ($P<0.001$), greater fat depth ($P<0.01$) and poorer residual

feed intakes (P<0.01) than LIMx steers. AAx steers had higher daily CH_4 outputs (P<0.001) but emitted less CH_4 per kg DM intake than LIMx steers (P<0.05). In conclusion, inclusion of nitrate reduced CH_4 emissions in growing beef cattle although the efficacy of nitrate was less than in previous work. When increased dietary lipid and nitrate inclusion were combined there was no evidence of an interaction between treatments and therefore combining different nutritional treatments to mitigate CH_4 emissions could be a useful means of achieving reductions in CH_4 while minimizing any adverse effects.

Keywords: beef cattle, greenhouse gas, methane, nitrate, dark grains.

Implications

The ability of individual nutritional strategies to reduce methane (CH_4) emissions from cattle is limited by potential adverse consequences such as reduction in fibre digestion for increased lipid or toxicity for added nitrate. The reduction in CH_4 emissions when dietary nitrate was fed was not influenced by the presence of lipid. Combining different nutritional strategies to mitigate CH_4 emissions could be a useful means of achieving reductions in CH_4 while minimizing any adverse effects on cattle health and performance.

Introduction

Methane emissions arising from the enteric fermentation of feed by ruminant livestock contribute significantly to greenhouse gas emissions. In the United Kingdom (Department of Energy and Climate Change, 2016), enteric CH_4 emissions were estimated to account for 23.8 Mt carbon dioxide equivalents or 48% of total greenhouse gas emissions from the agriculture sector in 2014. Strategies to mitigate

66 CH_4 emissions have been classified (Hristov *et al.,* 2013) as addressing enteric
67 fermentation, manure management or animal husbandry (where animal husbandry
68 included genetics, health and fertility).

69 Many nutritional strategies which target CH_4 emissions have been tested but
70 convincing evidence for long-term efficacy *in vivo* for many is lacking. Increasing
71 dietary lipid and inclusion of nitrate in the diet are effective mitigation strategies
72 (Hristov *et al.,* 2013) and their use has been recently reviewed (Martin *et al.,* 2010;
73 Patra, 2014; Lee and Beauchemin, 2014; Yang *et al.*, 2016). However, the extent to
74 which either strategy can be included in the diet is limited by potential adverse
75 effects: a reduction in fibre digestion and consequently feed intake from increased
76 lipid in the diet and nitrate / nitrite toxicity from adding nitrate. As the mechanisms by
77 which lipid (reduction in fermentable carbohydrate intake, inhibition of micro-
78 organisms; Martin *et al.,* 2010; Patra, 2013) and nitrate (alternative hydrogen
79 acceptor; Yang *et al.* 2016) reduce CH_4 emissions are different, it may be practically
80 useful to combine these mitigation strategies.

81 Klop *et al.* (2016) fed lipid and nitrate alone or in combination to dairy cows
82 and found no evidence for any negative interactions between strategies for either
83 CH_4 emissions or animal performance. Similarly in non-lactating dairy cows
84 (Guyader *et al.,* 2015), there were no interactions between nitrate and tea saponins.
85 Interactions between mitigation strategies have not been explored to date in beef
86 cattle. The main hypotheses addressed in this study were that the effects on CH_4
87 emissions of increasing lipid or including nitrate in diets of finishing beef cattle would
88 be additive and that there would be no adverse effects upon animal performance.
89 The nutritional strategies used were based on those reported previously (Troy *et al.,*
90 2015; Duthie *et al.,* 2016).

Materials and Methods

This experiment was conducted at Scotland's Rural College (SRUC) Beef and Sheep Research Centre in Edinburgh in 2014. The experimental protocol was approved by SRUC's Animal Welfare and Ethical Review Body, the Animal Experiments Committee and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986.

Experimental design, animals and diets

The experiment was a 2 × 4 factorial (breed × dietary treatment) design. The basal diet contained 550 forage (grass and whole crop barley silages): 450 concentrate (g/kg DM). The four dietary treatments were assigned according to a 2 x 2 factorial arrangement where te control diet (CTL) contained rapeseed meal as the main protein source which was replaced either with nitrate (NIT, 21.5 g nitrate/kg DM) or maize distillers dark grains (MDDG) to increase diet lipid concentration or both nitrate and MDDG (COMB). Forage to concentrate ratio was maintained constant by varying the amounts of barley included in the diets. Nitrate was added in the form of calcium ammonium nitrate (Calcinit; Yara, Oslo, Norway). The ingredient and nutritional composition of each dietary treatment are given in Table 1. The steers were offered diets *ad libitum*. Feed samples were analysed for DM, ash, CP, ADF, NDF, starch and ether extract (Ministry of Agriculture Fisheries and Food, 1992), and gross energy (GE) by adiabatic bomb calorimetry.

The 80 cross-bred steers (13 to 15 months of age at start of performance trial) used were from a rotational cross between pure-bred Aberdeen Angus or Limousin sires and cross-bred dams of those genotypes and are referred to as AAx and LIMx,

116 respectively. Thus, 20 steers (10 of each breed) were allocated to each dietary
117 treatment. To avoid animals not adapted to nitrate gaining access to dietary nitrate,
118 each treatment was allocated to one pen (four pens in total). Treatments were
119 balanced for sire within each breed and BW at the start of the experiment. Fresh
120 water was provided *ad libitum* using a water trough, and diets were offered using 32
121 electronic feeders (eight per pen, HOKO, Insentec, Marknesse, The Netherlands) to
122 record individual animal feed intakes. All steers were bedded on wood fibre and
123 sawdust to ensure that consumption of bedding did not contribute to nutrient intake.
124 Steers were adapted to the experimental diets in two stages. In stage one
125 (days -64 to -36 in relation to the start of the performance test on day 0), the animals
126 were all adapted to the control diet and trained to use the electronic feeders. In stage
127 two (days –35 to 0), steers were adapted to the treatments over a 5 week period.
128 Dietary treatments (nitrate and MDDG) were progressively incorporated into the NIT,
129 MDDG and COMB treatments at 25% (day -35), 50% (day -28), 75% (day -21) and
130 100% (day -14) of the final dietary inclusion.
131
132 *Blood met-haemoglobin (MetHb) measurements*
133 Blood MetHb is formed when nitrite, arising from reduction of nitrate in the rumen,
134 reacts with haemoglobin to form MetHb which is incapable of oxygen transport and
135 responsible for acute toxicity (Bruning-Fann and Kaneene, 1993). Blood MetHb
136 concentrations were monitored in all steers receiving dietary nitrate. Blood samples
137 were taken 3 h after fresh feed was offered, when MetHb concentrations were
138 expected to be greatest (van Zijderveld *et al.,* 2010). Samples were taken on days -
139 34 (25%), -27 (50%), -13 (100%), -6 (100%) and 1 (100%) where 100% is the final
140 dietary inclusion of nitrate. Blood samples were taken from the caudal vein into two

evacuated tubes (Vacuette 9 ml LH Lithium Heparin, Vacuette, Griener Bio One Ltd., Gloucestershire, UK). The samples were immediately combined to give one tube from which air was excluded, sealed and kept on ice until blood MetHb concentrations were measured within 2 h of sampling by co-oximetry (Stat Profile Critical Care Xpress, Nova Biomedical U.K., Cheshire, UK).

56 day performance test

After full adaptation to the experimental diets, performance and feed efficiency were characterised for all steers over a 56 day test period (days 0 to 56). Steers were maintained under controlled conditions, where group sizes within the pen remained constant. Individual DM intake (DMI, kg/day) was recorded for each animal using the electronic feeding equipment and BW was measured weekly, before fresh feed was offered, using a calibrated weigh scale. For all steers, ultrasonic fat depth was obtained at the 12th/13th rib at the start (FD0) and end (FD1) of the 56 day test using industry-standard equipment (Aloka 500, BCF Technology LTD, UK). Images were analysed using Matrox Inspector 8 software (Matrox Video and Imaging Technology Europe Ltd., Middlesex, UK).

Respiration chamber measurements

Seventy-two of the steers, balanced for breed and treatment were chosen for respiration chamber measurements. The steers remained on the same diets and in the same pens as described above prior to entering the respiration chamber facility. The steers were allocated to six respiration chambers over a 12 week period using a 4 (dietary treatment) x 6 (chamber) randomised block design which was replicated three times such that each dietary treatment was measured in each respiration

chamber three times over the 12 week period. Prior to entering the respiration chambers the steers were housed in training pens, identical in size and shape to the pens inside the chambers, for a period of one week, to adapt to individual housing. The steers were allocated to chambers to minimise variation in BW on entry into the respiration chambers between blocks; thus the heaviest steers for each treatment were included in the first block. The steers remained in the respiration chambers for 3 days, during which time they were fed *ad libitum* once daily. Data for DMI during the 3 day chamber measurement period were averaged per animal. One chamber malfunctioned from weeks 1 to 6, which resulted in the requirement for a thirteenth week of chamber analysis to obtain measurements from each of 72 steers. Full details of the methods are described in Troy *et al.* (2015).

Rumen sampling and analysis

Rumen fluid samples were taken to assess long term changes in rumen volatile fatty acid (VFA) molar proportions from each animal on five occasions: before adaptation to dietary treatments (day -42); during adaptation (day -28); pre-performance test (day -11); end of performance test (day 56); immediately after leaving respiration chambers. Samples, approximately 50 ml rumen liquid, were taken before fresh feed was offered, by inserting a stomach tube (16 × 2700 mm Equivet Stomach Tube, JørgenKruuse A/S, Langeskov, Denmark) nasally and aspirating manually. This liquid was filtered through two layers of muslin. A 5 mL sample of the filtered liquid was deproteinised by adding 1 mL metaphosphoric acid (215 g/l) and 0.5 ml methylvaleric acid (10 g/l) was added as an internal standard. These samples were stored at -20 °C between collection and analysis. Volatile fatty acid concentrations were determined by high performance liquid chromatography (Rooke *et al.* 1990).

Calculations and statistical analyses

Data from one steer from the 56 day test period was discarded as the steer was removed from the trial for health reasons unconnected to the diets and treatments imposed, leaving data from 79 steers available for analysis. Growth was modelled by linear regression of BW against test date, to obtain average daily gain (ADG), mid-test BW (mid-BW) and mid-test metabolic BW (mid-MBW, $BW^{0.75}$). Mean DMI over the 56 day period was expressed as kg per day or as a proportion of mid-BW and mid-MBW. Feed conversion ratio (FCR) was calculated as average DMI (kg/day) / ADG. Residual feed intake (RFI) was calculated as deviation of actual DMI (kg/day) from DMI predicted based on linear regression of actual DMI on ADG, mid-MBW and FD1 (Basarab *et al.,* 2003).

Statistical analyses of performance data were conducted using the mixed procedure of SAS software (SAS 9.3 for Windows; SAS Inst. Inc., Cary, USA) with the fixed effects of breed, nitrate and lipid. In addition, in the analysis of FD1 and FD2, the deviation from the breed mean of FD0 was included as a covariate. In the analysis of the respiration chamber data, fixed effects were breed, nitrate and lipid, while the random effects were week of chamber measurement and chamber. The interactions, breed × nitrate, nitrate × lipid, breed × lipid and breed × nitrate × lipid were included as fixed effects in each model when these effects proved significant ($P < 0.05$).

Changes in MetHb concentration were analysed using a repeated measures design where the fixed effects were breed, lipid and time and their interactions. As significant time x breed x lipid interactions were found, a two factor (breed x lipid) ANOVA was then performed for each time to characterise this interaction. Where a

significant breed x lipid interaction was detected, differences between individual treatments were characterized using LSD.

Molar proportions of VFA in rumen fluid when steers left the respiration chamber were analysed using fixed effects of breed, nitrate and lipid and their interactions with week of measurement as a random effect. For differences in the ratio, acetate to propionate, between samples taken at different stages of the experiment, a split plot ANOVA was used where sample was a split plot within steer and the effects of breed, nitrate, lipid and sample and their interactions were included in the model. Data for samples taken prior to introduction of dietary treatments (day -42), were included as a covariate in the model to control for pre-existing differences between steers in VFA pattern. For all analyses data are reported as means with their standard errors of the mean unless indicated otherwise. Probability values of $P<0.05$ were deemed to be significant, while probability values $P>0.05$ and $P<0.1$ were deemed to indicate a tendency.

Results

Met-haemoglobin response to dietary nitrate

MetHb concentrations were low (<1% total haemoglobin (Hb), Figure 1) when nitrate was included at 25 and 50% of the maximum inclusion. Adding 100% nitrate increased MetHb concentrations ($P<0.001$); mean values were greater on day -6 (7.9% total Hb) than on day -13 (2.9% total Hb) or day 1 (2.2% total Hb). There was a significant time x breed x nitrate interaction ($P<0.01$). On day -13, Met-Hb concentrations were greater (P<0.01) on the COMB than the NIT treatment. However, on day -6, AAx steers on treatment COMB had greater MetHb concentrations (4.1% SEM 0.65) than other treatments (1.6% SEM 0.25). For

241 individual steers, the highest values for MetHb concentration recorded were 13.0,
242 20.5 and 7.6 % total Hb on days -13, -6 and 1 respectively. Clinical signs of toxicity
243 are considered to become apparent at MetHb values of 30 to 40% total Hb (Bruning-
244 Fann and Kaneene, 1993).
245
246 As there were no interactions between breed and dietary treatment for any
247 measurement, for clarity, results in Tables 2 to 4 are presented as main effects of
248 breed and dietary treatment.
249
250 *Performance traits*
251 At the start of the experiment the treatments were balanced for age and BW. Thus,
252 age at the start of the test period (AgeST) and Mid-BW did not differ across dietary
253 treatments (P>0.05, Table 2). DMI was not affected by the inclusion of nitrate or lipid
254 (P>0.05). However, steers receiving dietary nitrate achieved poorer ADG throughout
255 the 56 day test (P<0.01). The inclusion of nitrate or lipid did not affect fat depth at the
256 end of the 56 day test (FD1; P>0.05). Steers receiving dietary nitrate were less
257 efficient (greater FCR; P<0.05) than those not receiving nitrate, although this
258 difference was not observed for RFI. In contrast dietary lipid did not affect feed
259 efficiency (P>0.05). AgeST and Mid-BW did not differ between breeds (P > 0.05).
260 AAx steers achieved greater ADG compared to LIMx steers (P<0.01). DMI was
261 greater in AAx compared to LIMx steers, whether expressed daily (P<0.001), or as a
262 proportion of BW (P<0.001). FD1 was greater in AAx compared to LIMx steers (P <
263 0.05). Due to the higher levels of DMI and FD1, AAx steers were less efficient with
264 greater RFI scores than LIMx steers (P<0.01).
265

Methane and hydrogen emissions

Steers receiving treatments which included nitrate produced less CH_4 and more hydrogen (Table 3) than those treatments without added nitrate, expressed on either a daily or on a DMI basis (P<0.001). Increasing the lipid content of the diets had no effect on CH_4 or H_2 emissions. When expressed on a GE basis, CH_4 emissions (kJ/MJ GE intake) were reduced when lipid was included in the diet. There were no significant interactions between inclusion of nitrate and lipid on CH_4 emissions on either a daily (P=0.59) or DMI (P=0.82) basis. There were no differences in CH_4 emissions when calculated /kg ADG for any nutritional treatment. AAx steers were heavier than the LIMx steers (P<0.01) and had a higher DMI during the chamber period (P<0.001). Therefore, they produced more CH_4 on a daily basis (P<0.001). In contrast, the LIMx steers produced more CH_4 on a DMI basis (P<0.05). Breed had no effect on H_2 emissions on either a daily or DMI basis.

Volatile fatty acid molar proportions

When nitrate was included in the diets, acetate molar proportions were greater (P<0.001), those of propionate less (P<0.01) and therefore acetate to propionate ratio (APR) greater (P<0.001) in rumen samples taken when steers left the respiration chambers (Table 4). Increasing the lipid concentration of the diet also increased (P < 0.05) the molar proportions of acetate. There were greater molar proportions of acetate (P<0.01) and lesser propionate proportions (P<0.05) in LIMx than AAx steers. The APR ratio differed (P<0.001) in samples taken at different times during the experiment (Figure 2), increasing as the experiment progressed. APR in samples taken prior to introduction of dietary treatments was a significant covariate (P<0.01) in the model indicating that individual steer VFA pattern prior to

inclusion of treatments influenced VFA pattern throughout the experiment. The main effects of breed and treatment across the experiment were consistent with samples taken when steers left the respiration chambers (Table 4). Thus, LIMx steers had greater APR than AAx steers (P<0.05) and inclusion of both nitrate (P<0.001) and lipid (P<0.05) increased APR.

Discussion

This study extended those of Troy *et al.* (2015) and Duthie *et al.* (2016) by using a factorial design to investigate whether the effects of individual treatments to reduce CH_4 emissions were additive and to characterize the consequences for animal performance. The diets were formulated from feedingstuffs practical for use in beef cattle systems. To maintain CP constant, MDDG and nitrate replaced dietary rapeseed meal. Thus the increase in dietary lipid concentration achieved with MDDG was modest. However, this was representative of what is practically achievable using by-product feeds. To achieve higher lipid concentrations, it would have been necessary to use materials from which oil could have been extracted for human food consumption. Since a main objective was to investigate the combined effects of lipid and nitrate, the COMB treatment inevitably contained a higher CP concentration than the other treatments. However, there were no interactions for any performance measurement between lipid and nitrate and thus no adverse effects of the higher CP.

Diet effects

Nitrate. Addition of nitrate to the diet reduced CH_4 emissions, a consistent finding across many studies (see review by Lee and Beauchemin, 2014 and more recent

316 studies including: Newbold *et al.,* 2014; Guyader *et al.,* 2015, 2016; Lee *et al.,* 2015;
317 Troy *et al.,* 2015; Veneman *et al.,* 2015; Klop *et al.,* 2016). Unexpectedly, the
318 reduction in CH_4 from added nitrate was only 10% or 2.2 g/kg DM less than for diets
319 not containing nitrate. As H_2 consumed in reduction of 1 mole nitrate is equivalent to
320 that used in formation of 1 mole CH_4, the reduction in CH_4 was only 45% of the
321 theoretical maximum possible for the dietary nitrate inclusion. A 17% (4.4 g/kg DMI)
322 reduction in CH_4 yield (80% of theoretical maximum) was reported by Troy *et al.*
323 (2015) who used very similar experimental conditions to the present experiment. The
324 meta-analysis of Lee and Beauchemin (2014) predicted that the amount of nitrate
325 used would have reduced CH_4 emissions by 18%. A more recent analysis of the
326 efficacy of nitrate, including the studies cited above (Rooke *et al.,* 2016) found that a
327 mean inclusion of 21g nitrate / kg DMI, reduced mean CH_4 (g/kg DMI) by 21%.
328 The lower than expected reduction in CH_4 by nitrate was accompanied by
329 reduced FCR in nitrate-fed animals. Again, the reduction in animal performance was
330 unexpected as in none of the studies reviewed by Lee and Beauchemin (2014) or
331 most recent studies (Li *et al.,* 2013; de Raphelis-Soissan *et al.,* 2014; Lee *et al..*
332 2015; Veneman *et al.,* 2015; Duthie *et al.,* 2016; Klop *et al.,* 2016) has animal
333 performance been compromised by inclusion of nitrate. However, Guyader *et al.*
334 (2016) reported reduced fat and protein corrected milk yield when both nitrate and
335 extruded linseed were added to the diet and Hegarty *et al.* (2016) in a feedlot study
336 using high grain diets (700 g / kg DM) reported reduced DMI, ADG and FCR when
337 nitrate replaced urea. In the current experiment, since DMI was not changed when
338 nitrate was added to the diet, then the poorer FCR must have been due to alterations
339 in nutrient supply or utilization.

Although MetHb concentrations of greater than 15% total Hb were observed during adaptation to nitrate, these were isolated occurrences and were substantially less than the 30 to 40% total Hb considered to lead to clinical toxicity (Bruning-Fann and Kaneene, 1993). As reduced performance is the most likely response to mild or subclinical toxicity, then this cannot be excluded as a reason for the poorer FCR in nitrate-fed animals. Another possibility is that rumen microbial protein synthesis and therefore host animal amino acid supply may have been less than expected. The reduction in CH_4 attributed to nitrate was only 45% of the theoretical maximum. This implies that there was a corresponding reduction in the conversion of nitrate to ammonia. When the degradable protein supply to the rumen (ERDP) was estimated according to AFRC (1993) for diets CTL and NIT, ERDP supply was greater than requirement (1.14 and 1.18-fold respectively; 9.9 and 10.6 g ERDP/MJ fermentable ME, FME). When the ERDP supply from nitrate was reduced to 0.45 of that supplied by nitrate on diet NIT, ERDP supply was reduced to 0.99 of requirement or 9.0 g ERDP/MJ FME. However, estimated metabolisable protein supply to the animal was in excess of requirement (1.40 (CTL), 1.35 (NIT) and 1.30 fold (NIT with reduced ERDP supply) and therefore overall no reduction in performance as a result of reduced ERDP supply would have been expected as a consequence of a reduction in conversion of nitrate to ammonia. A further possibility is that as nitrate, a non-protein nitrogen source of ERDP replaced rapeseed meal in the diet then protein supply may have been impaired. However, this is unlikely as the reduction in FCR was a main effect of nitrate and so was also observed in diet COMB where both nitrate and MDDG were included in the diet and protein supply to both rumen and animal would have been in excess.

364 *Lipid.* Because inclusion of MDDG in diets was limited by the need to avoid excess
365 CP intake, the increase in lipid content of the diet was modest (12 g/kg DMI) and the
366 overall reduction in CH_4 emissions was not significant (P=0.12) on a g/kg DMI basis
367 and only became significant on a kJ/MJ basis. However, the numerical decrease in
368 CH_4 emissions (g/kg DMI) of 4% (3% for a 10 g/kg diet DM increase in lipid) was
369 consistent with the meta-analysis of Martin *et al.* (2010) of a reduction in CH_4 (g/kg
370 DMI) of 3.8% for a 10 g/kg DM addition of supplementary fat.
371 *Nitrate and lipid.* A primary aim of the experiment was to investigate whether the
372 effectiveness of different nutritional methods for reducing CH_4 were additive. Since
373 the interaction between treatments was not significant (*P*=0.82) for CH_4 yield (g/kg
374 DMI) then there was no evidence that the effects of nitrate and lipid were not
375 additive. Most *in vivo* studies investigating different strategies for reducing CH_4 have
376 either compared different treatments (e.g. van Zijderveld *et al.,* 2011a; El-Zaiat *et al.,*
377 2014) or the combined effects of treatments (van Zijderveld *et al.,* 2011b; Li *et al.,*
378 2013; Caetano *et al.,* 2016) but have not adopted the factorial design necessary to
379 quantify interactions between treatments. Van Zijderveld *et al.* (2010) compared
380 nitrate and sulphate when fed to sheep and found the effects of treatments on CH_4
381 emissions were additive. Similarly de Raphelis-Soissan *et al.* (2014) fed nitrate and
382 *Propionibacterium acidipropionici* to sheep and Klop *et al.* (2016) nitrate and
383 docosahexaenoic acid to dairy cows and again treatment effects on CH_4 emissions
384 were additive. Thus in agreement with the current study, there is no evidence that
385 the effects of nutritional mitigation strategies for reducing CH_4 are not additive.
386 The additive nature of mitigation treatments is of practical importance. The
387 extent to which lipid-containing feeds can be incorporated into diets is limited by the
388 need to avoid reductions in fibre digestion when lipid concentrations are greater than

389 70 g/kg DM (Patra 2013). During adaptation to nitrate, in both the current experiment
390 and Duthie *et al*. (2016), blood Met-Hb concentrations did not increase until 15 g
391 nitrate / kg diet DM was included in the diet. This is likely because the apparent
392 efficiency of CH_4 reduction by nitrate decreases as nitrate inclusion increases (Leng
393 2014). Thus, potential adverse effects of mitigation strategies such as toxicity and
394 impaired animal performance could be avoided by feeding lesser amounts of nitrate
395 than in the present experiment.

396 *Breed effects*

397 Total CH_4 emissions were greater in AAx than LIMx steers as noted before (Rooke
398 *et al.,* 2014) primarily because of greater DMI. However unlike Rooke *et al.* (2014),
399 CH_4 yield (g/kg DMI) was lower in AAx than LIMx steers. This was probably because
400 of a faster rumen turnover rate in the AAx steers, an effect that has been well
401 documented and included in empirical prediction equations for CH_4 yield (Sauvant
402 and Giger-Reverdin 2009; Ramin and Huhtanen 2013). Similarly the smaller
403 proportion of acetate in rumen fluid samples from AAx steers was consistent with the
404 meta-analysis of Nozière *et al.* (2011). In the performance trial, the greater DMI of
405 AAx steers noticed when CH_4 was measured was also evident and resulted in
406 greater ADG in AAx steers. However when the RFI of the steers was assessed, the
407 LIMx steers were more efficient (smaller RFI) than the AAx steers. The lower CH_4
408 emissions (g/kg DMI) and higher propionic molar proportion achieved with the AAx
409 steers is not consistent with the differences in RFI. There is no obvious reason for
410 these discrepancies but it should be noted that the performance and respiration
411 chambers measurements were made sequentially.

412 In conclusion, inclusion of nitrate in the diet reduced CH_4 emissions in
413 growing beef cattle although the efficacy of nitrate was less than in previous work

414 (Troy *et al.,* 2015). Whereas Duthie *et al.* (2016) recorded no changes in animal
415 performance when nitrate was fed, in the present experiment, growth rate and feed
416 conversion ratio were poorer when nitrate was included in the diet. However, when
417 both increased dietary lipid and nitrate inclusion were combined there was no
418 evidence of any interaction between treatments in CH_4 emissions or performance
419 traits. Therefore, combining different nutritional treatments to mitigate CH_4 emissions
420 could be a useful means of achieving reductions in CH_4 emissions without adverse
421 effects.
422
423

424 **Acknowledgments**

425 The authors are grateful to the technical staff at the Beef Research Centre for their
426 skilled assistance during the experiment. This research was funded by AHDB Beef
427 and Lamb, the Scottish Government and by DEFRA and the devolved
428 administrations through the UK Agricultural Greenhouse Gas Inventory Research
429 Platform.

430 **References**

431 AFRC (1993) Energy and protein requirements of ruminants. An advisory manual prepared
432 by the AFRC Technical Committee on Responses to Nutrients. CAB International,
433 Wallingford, UK.
434 Basarab JA, Price MA, Aalhus JL, Okine EK, Snelling WM and Lyle KL 2003. Residual feed
435 intake and body composition in young growing cattle. Canadian Journal of Animal Science
436 83, 189-204.
437 Bruning-Fann, CS and Kaneene JB 1993. The effects of nitrate, nitrite, and N-nitroso
438 compounds on animal health. Veterinary and Human Toxicology 35, 237–253.

439 Caetano M, Wilkes M, Pitchford W, Lee S and Hynd P 2016. Efficacy of methane-reducing
440 supplements in beef cattle rations. Animal Production Science 56, 276-281.

441 Department of Energy and Climate Change 2016. 2014 UK Greenhouse Gas Emissions
442 Final Figures – Statistical release. London: Department of Energy and Climate Change.

443 de Raphelis-Soissan V, Li L, Godwin IR, Barnett MC, Perdok HB and Hegarty RS 2014. Use
444 of nitrate and Propionibacterium acidipropionici to reduce methane emissions and increase
445 wool growth of Merino sheep. Animal Production Science 54, 1860-1866.

446 Duthie C-A, Rooke JA, Troy S, Hyslop JJ, Ross DW, Waterhouse A and Roehe R 2016.
447 Impact of adding nitrate or increasing the lipid content of two contrasting diets on blood
448 methaemoglobin and performance of two breeds of finishing beef steers. Animal 10, 786-
449 795.

450 El-Zaiat HM, Araujo RC, Soltan YA, Morsy AS, Louvandini H, Pires AV, Patino HO, Correa
451 PS and Abdalla AL 2014. Encapsulated nitrate and cashew nut shell liquid on blood and
452 rumen constituents, methane emission, and growth performance of lambs. Journal of Animal
453 Science 92, 2214-2224.

454 Guyader G, Eugène M. Doreau M, Morgavi DP, Gérard C, Loncke C and Martin C 2015.
455 Nitrate but not tea saponin feed additves decreased enteric methane emissions in
456 nonlactating cows. Journal of Animal Science 93, 5367-5377.

457 Guyader J, Doreau M, Morgavi DP, Gerard C, Loncke C and Martin C 2016. Long-term
458 effect of linseed plus nitrate fed to dairy cows on enteric methane emission and nitrate and
459 nitrite residuals in milk. Animal 10, 1173-1181.

460 Hegarty RS, Miller J, Oelbrandt N, Li L, Luijben JPM, Robinson DL, Nolan JV and Perdok
461 HB 2016. Feed intake, growth, and body and carcass attributes of feedlot steers
462 supplemented with two levels of calcium nitrate or urea. Journal of Animal Science 94, 5372-
463 5381

464 Hristov AN, Oh J, Lee C, Meinen R, Montes F, Ott T, Firkins J, Rotz A, Dell C, Adesogan A,
465 Yang W,Tricarico J, Kebreab E, Waghorn G, Dijkstra J and Oosting S 2013. Mitigation of
466 greenhouse gas emissions in livestock production – A review of technical options for non-
467 CO2 emissions. Eds Gerber PJ, Henderson B and Makkar HPS. FAO Animal Production
468 and Health Paper No. 177. FAO, Rome, Italy.

469 Klop G, Hatew B, Bannink A and Dijkstra J 2016. Feeding nitrate and docosahexaenoic acid
470 affects enteric methane production and milk fatty acid composition in lactating dairy cows.
471 Journal of Dairy Science 99, 1161-1172.

472 Lee C, Araujo R, Koenig K and Beauchemin K 2015. Effects of encapsulated nitrate on
473 enteric methane production and nitrogen and energy utilization in beef heifers. Journal of
474 Animal Science 93, 2391-2404.

475 Lee C and Beauchemin KA 2014. A review of feeding supplementary nitrate to ruminant
476 animals: nitrate toxicity, methane emissions, and production performance. Canadian Journal
477 of Animal Science 94, 557-570.

478 Leng RA 2014. Interactions between microbial consortia in biofilms: a paradigm shift in
479 rumen microbial ecology and enteric methane mitigation. Animal Production Science 54,
480 519-543

481 Li L, Silveira C, I, Nolan J, V, Godwin I, Leng R and Hegarty R 2013. Effect of added dietary
482 nitrate and elemental sulfur on wool growth and methane emission of Merino lambs. Animal
483 Production Science 53, 1195-1201.

484 Martin C, Morgavi DP and Doreau M 2010. Methane mitigation in ruminants: from microbe to
485 the farm scale. Animal 4, 351-365.

486 Ministry of Agriculture Fisheries and Food 1992. Analysis of Agricultural Materials, 2nd Ed.
487 Her Majesty's Stationary Office, London.

488 Newbold JR, van Zijderveld SM, Hulshof RBA, Fokkink WB, Leng RA, Terencio P, Powers
489 WJ, van Adrichem PSJ, Paton ND and Perdok HB 2014 The effect of incremental levels of

dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. Journal of Animal Science 92, 5032-5040.

Noziere P, Glasser F and Sauvant D 2011. In vivo production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. Animal 5, 403-414.

Patra AK 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livestock Science 155, 244-254.

Patra AK 2014. A meta-analysis of the effect of dietary fat on enteric methane production, digestibility and rumen fermentation in sheep, and a comparison of these responses between cattle and sheep. Livestock Science 162, 97-103.

Ramin M and Huhtanen P 2013. Development of equations for predicting methane emissions from ruminants. Journal of Dairy Science 96, 2476-2493.

Rooke JA, Borman AJ and Armstrong DG 1990. The effect of inoculation with Lactobacillus plantarum on fermentation in laboratory silos of herbage low in water-soluble carbohydrate. Grass and Forage Science 45, 143-152.

Rooke JA, Wallace RJ, Duthie C-A, McKain N, de Souza SM, Hyslop JJ, Ross DW, Waterhouse T and Roehe R 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. British Journal of Nutrition 112, 398–407

Rooke JA, Miller G, Flockhart J, McDowell M and MacLeod M 2016. Nutritional strategies to reduce enteric methane emissions. climateXchange Retrieved on 17 March 2017, from http://www.climatexchange.org.uk/reducing-emissions/emissions-livestock-production/

Sauvant D and Giger-Reverdin S 2009. Modelling of digestive interactions and methane production in ruminants. Productions Animales 22, 375-384.

515 Troy S, Duthie CA, Hyslop J, Roehe R, Ross D, Wallace R, Waterhouse A and Rooke J
516 2015. Effectiveness of nitrate addition and increased oil content as methane mitigation
517 strategies for beef cattle fed two contrasting basal diets. Journal of Animal Science 93, 1815-
518 1823.

519 van Zijderveld S, Gerrits W, Apajalahti J, Newbold J, Dijkstra J, Leng R and Perdok H 2010.
520 Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane
521 production in sheep. Journal of Dairy Science 93, 5856-5866.

522 van Zijderveld SM, Fonken B, Dijkstra J, Gerrits WJJ, Perdok HB, Fokkink W and Newbold
523 JR 2011a. Effects of a combination of feed additives on methane production, diet
524 digestibility, and animal performance in lactating dairy cows. Journal of Dairy Science 94,
525 1445-1454.

526 van Zijderveld SM, Gerrits WJJ, Dijkstra J, Newbold JR, Hulshof RBA and Perdok HB
527 2011b. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows.
528 Journal of Dairy Science 94, 4028-4038.

529 Veneman JB, Muetzel S, Hart KJ, Faulkner CL, Moorby JM, Perdok HB and Newbold CJ
530 2015. Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and
531 Animal Productivity in Dairy Cows? Plos One 10, e0140282.

532 Yang CJ, Rooke JA, Cabeza I and Wallace RJ 2016. Nitrate and Inhibition of Ruminal
533 Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane
534 Emissions from Ruminant Livestock. Frontiers in Microbiology 7, 132.
535 DOI:**10.3389**/fmicb.2016.00132.

Table 1. Ingredient and chemical composition (g/kg DM) of the experimental diets

Treatment	Control	Nitrate	MDDG	Combined
Ingredient Composition				
Barley	336	388	289	263
Grass silage	210	211	209	210
Whole crop barley silage	347	347	346	346
Rapeseed meal	79	0	0	0
Calcinit[1]	0	25	0	24
Maize distiller's dark grains	0	0	128	127
Molasses	19	20	19	19
Minerals[2]	9	9	9	9
Chemical Composition				
DM, g/kg	533	531	533	533
Ash	52	48	51	51
CP	135	141	136	162
ADF	184	166	184	183
NDF	308	295	317	313
Starch	281	308	264	250
Ether extract	25.0	23.4	36.7	35.9
GE, MJ/kg DM	18.1	17.6	18.5	18.0
Estimated ME, MJ/kg DM	11.7	11.5	12.0	11.7

[1]Contained (g/kg DM): nitrate, 757; Ca, 225.

[2]Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; (µg/kg): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500.

Table 2. Effect of breed and dietary treatment on growth, fat depth, feed intake and feed efficiency of Aberdeen Angus- (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB)

	Breed		Significance[2]	Treatment					Significance[2]	
Treatment	AAx	LIMx		CTL	NIT	MDDG	COMB	SEM[1]	Nitrate	Lipid
AgeST (days)	417	411	NS	414	414	413	415	5.3		
Mid-BW (kg)	542	539	NS	547	543	538	534	17.5		
Mid-MBW ($kg^{0.75}$)	112	112	NS	113	112	112	112	2.7		
ADG (kg/day)	1.75	1.56	**	1.74	1.54	1.72	1.63	0.076	**	
DMI (kg/day)	12.15	11.07	***	11.78	11.43	11.76	11.47	0.425		
DMI/BW(g/kg)	22.44	20.58	***	21.60	21.08	21.90	21.47	0.483		
DMI/MBW($g/kg^{0.75}$)	108.1	99.1	***	104.3	101.6	105.3	103.1	2.31		
FCR (kg, kg)	7.02	7.21	NS	6.85	7.52	6.90	7.18	0.269	*	
RFI (kg)	0.24	-0.24	**	-0.08	0.06	-0.02	0.04	0.231		
FD1 (mm)[3]	9.14	8.05	**	8.40	8.86	8.81	8.31	0.663		

[1]SEM for 10 observations.

AgeST, Age at start of test; Mid-BW, mid-test BW; Mid-MBW, mid-test metabolic BW; ADG, average daily gain at the end of the 56 day test; FCR, feed conversion ratio; RFI, residual feed intake; FD1, fat depth at the 12/13th rib at the end of the 56 day test.

[2] There were no significant ($P>0.05$) interactions between breed and dietary treatment or between nitrate and lipid.

[3]Deviation from breed mean of FD0 (measured at start of 56 day performance test) fitted as covariate.

*$P<0.05$; **$P<0.01$; $P<0.001$

Table 3. Effect of breed and dietary treatment on methane and hydrogen emissions of Aberdeen Angus-sired (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB)

	Breed			Treatment					Significance[2]	
	AAx	Limx	Significance	CTL	NIT	MDDG	COMB	SEM[1]	Nitrate	Lipid
BW (kg)	669	648	**	677	650	652	655	9.5		
DMI										
kg/day	11.0	9.3	***	10.3	9.8	10.2	10.2	0.51		
g/kg BW	16.4	14.3	***	15.3	15.0	15.6	15.6	0.65		
Methane										
g/day	241	214	***	246	219	238	210	12.2	***	
g/kg DMI	22.0	23.2	*	24.0	22.1	23.4	20.9	0.94	***	
g/kg ADG[3]	154	163		161	163	161	147	7.9		
kJ/MJ GEI	67.5	71.1	*	73.3	69.5	70.2	64.2	2.9	**	*
Hydrogen										
g/day	0.86	0.67		0.45	0.99	0.40	1.04	0.095	***	
g/kg DMI	0.06	0.07		0.04	0.10	0.04	0.10	0.009	***	
kJ/MJ GEI	0.56	0.58		0.35	0.81	0.30	0.82	0.073	***	
H_2:CH_4 molar ratio	0.025	0.025		0.015	0.035	0.01	0.04	0.003	***	

DMI, DM intake; GEI, Gross Energy intake;

[1] SEM for 9 observations.

[2]There were no significant (P>0.05) interactions between breed and dietary treatments or between nitrate and lipid.

*P<0.05; **P<0.01; ***P<0.001

[3] Calculated from methane (g/kg DMI) and DMI and ADG from Table 2.

Table 4. Effect of breed and dietary treatment on VFA (mmol/mol) molar proportions in rumen fluid from Aberdeen Angus-sired (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB). Rumen samples taken when steers left respiration chambers

	Breed			Treatment					Significance[2]	
Treatment	AAx	Limx	Significance	CTL	NIT	MDDG	COMB	SEM[1]	Nitrate	Lipid
Acetate	672	689	***	664	685	676	696	7.4	***	*
Propionate	167	155	*	175	154	164	150	7.3	**	
Butyrate	126	123		124	127	125	123	5.8		
Valerate	13	11	†	13	12	12	10	1.0	†	†
Branched chain	34	35		40	34	29	34	2.8		**
Acetate: Propionate Ratio	4.0	4.5	**	3.9	4.5	4.2	4.7	0.33	***	

[1]SEM given for 9 observations.

[2]There were no significant interactions (P>0.05) between breed and treatments

†, P<0.1; *P<0.05; **P<0.01; ***P<0.001

Legends for Figures

Figure 1. Changes in blood met-haemoglobin (% total haemoglobin) during adaptation to nitrate-containing diets. Samples were obtained from cross bred Aberdeen Angus (AAx) or Limousin (LimX) steers offered diets containing nitrate alone (NIT) or nitrate and maize distillers dark grains (COMB). Blood samples were taken when 25% (day -34, where day 0 was the start of the performance test), 50% (day -27) and 100% (days -13, -6 and 1) of the dietary nitrate (100% = 21.5 g nitrate / kg DM) inclusion was offered.

Figure 2 Changes in the ratio (mol /mol) of acetate to propionate during experiment in rumen fluid from steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB). Samples were taken during adaptation to basal diet (Prelim, day -42); during introduction of experimental treatments (Adapt, day -28); prior to the start (Start, day -11) and at the end (End, day 56) of performance measurement and when steers left respiration chambers (Chamber).

Figure 1. Changes in blood met-haemoglobin (% total haemoglobin) during adaptation to nitrate-containing diets. Samples were obtained from cross bred Aberdeen Angus (AAx) or Limousin (LimX) steers offered diets containing nitrate alone (NIT) or nitrate and maize distillers dark grains (COMB). Blood samples were taken when 25% (day -34, where day 0 was the start of the performance test), 50% (day -27) and 100% (days -13, -6 and 1) of the dietary nitrate (100% = 21.5 g nitrate / kg DM) inclusion was offered.

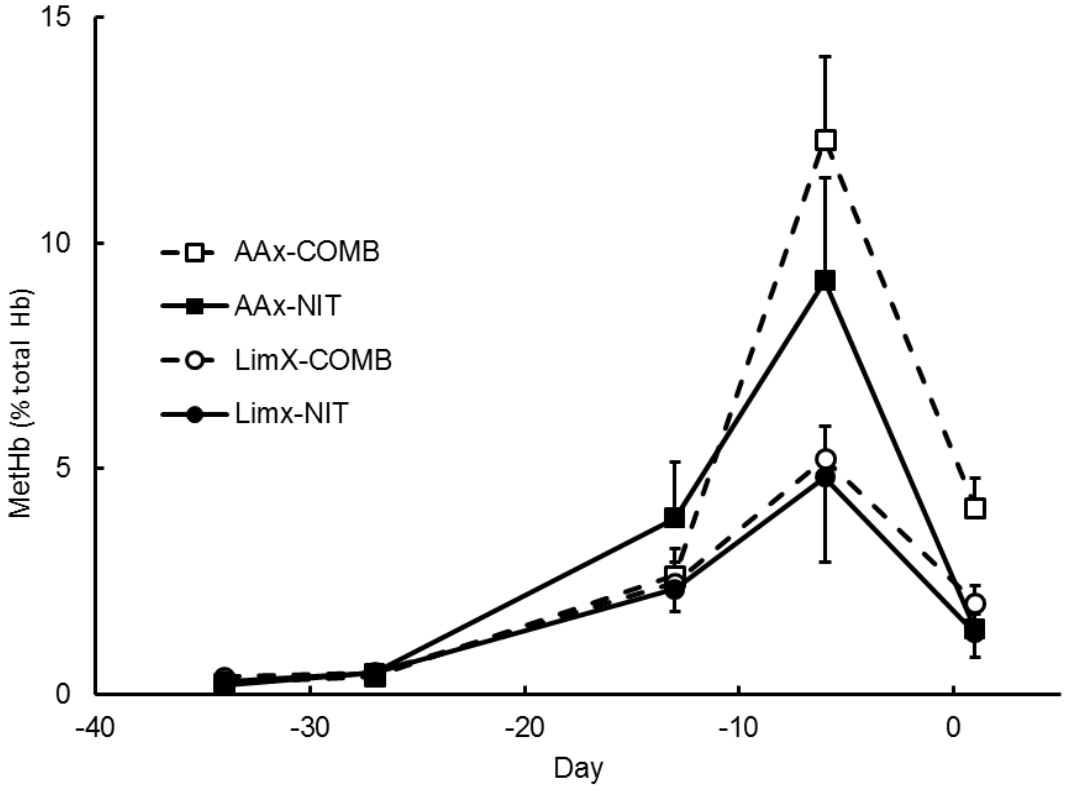

Figure 2 Changes in the ratio (mol /mol) of acetate to propionate during experiment in rumen fluid from steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB). Samples were taken during adaptation to basal diet (Prelim, day -42); during introduction of experimental treatments (Adapt, day -28); prior to the start (Start, day -11) and at the end (End, day 56) of performance measurement and when steers left respiration chambers (Chamber).

Deparment